

Isolation and characterization of strains of *Flavobacterium columnare* from Brazil

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Abstract

Flavobacterium columnare is an important pathogen of freshwater fish, implicated in skin and gill disease, often causing high mortality. An outbreak of skin disease in fingerling and adult Nile tilapia, *Oreochromis niloticus* (L.), cultivated in a recirculation system, was investigated. Four strains were isolated and characterized by biochemical reactions, enzyme production, fatty acid profile and analysis of the 16S-23S rDNA intergenic spacer region. All strains were identified as *F. columnare*. Experimental infection assays with one of these strains (BZ-5-02) were conducted and pathogenicity (by intramuscular route) was demonstrated in Nile tilapia and channel catfish, *Ictalurus punctatus* (Rafinesque). This is the first report of characterization of Brazilian strains of *F. columnare*.

Keywords: Brazil, characterization, fish disease, *Flavobacterium columnare*, isolation, *Oreochromis niloticus*.

Introduction

Flavobacterium columnare, a Gram-negative gliding bacterium, is the causative agent of columnaris disease. *Flavobacterium columnare* is an important

bacterial pathogen of freshwater fish (Decostere, Haesebrouck, Turnbull & Charlier 1999a). It is believed that the infection has a worldwide distribution, with economic losses associated with skin lesions and mortality (Bader, Shoemaker & Klesius 2003a).

Columnaris disease is characterized by white to yellow erosions in the tegument, necrosis of skin and gills, and sometimes results in a systemic infection (Stringer-Roth, Yunghans & Caslake 2002). Generally, outbreaks are associated with poor environmental conditions, such as stock densities, organic load and high temperature (Decostere *et al.* 1999a). Phenotypic and genetic variations among strains of *F. columnare* from distinct origins have been reported. These variations are found in colony morphology, pattern of biochemical reactions and variability in analysis of 16S rDNA by restriction fragment length polymorphism (Decostere, Haesebrouck & Devriese 1998; Triyanto, Kumamaru & Wakabayashi 1999; Triyanto & Wakabayashi 1999; Arias, Welker, Shoemaker, Abernathy & Klesius 2004).

In Brazil, many outbreaks of skin disease in fish that resemble columnaris disease have been observed, to date, without a laboratory confirmation. The main fish species involved is the Nile tilapia, *Oreochromis niloticus* (L.), with high mortality rates in fry production units (hatcheries) and floating cage systems.

The aim of this study was to report the isolation and characterization of Brazilian strains of *F. columnare*, from fingerlings and adult tilapia.

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Materials and methods

Fish and outbreak characterization

Two sequential outbreaks (with a month interval) occurred in a commercial hatchery of Nile tilapia, which produces an average of 600 000 fingerlings per month using a recirculation system. The main lesions observed were fin necrosis and skin ulceration in both fingerlings and adult fish with a significant daily mortality rate. Ten fish and 30 fingerlings (one collection from each outbreak) with typical clinical signs were collected from the affected ponds and sent alive to the laboratory in plastic transportation bags, with oxygen supply.

Isolation and biochemical characterization of *F. columnare* strains

Samples obtained from lesions were streaked onto Shieh agar (Shieh 1980), supplemented with polymyxin B (10 U mL⁻¹). The plates were incubated at 30 °C for 48 h and suspect colonies were selected for further analysis. The characterization was based on colony morphology, Gram staining, and standard biochemical tests as described by Griffin (1992) and Shamsudin & Plumb (1996) and compared with the reference strains *F. columnare* (ATCC 23463^T), *F. aquatile* (ATCC 11947^T) and *F. johnsoniae* (ATCC 17061^T).

Enzymatic activity and fatty acid methyl esters profile

Enzyme production of the isolated strains was determined by API ZYM kit (API ZYM 25 200, bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Hsu-Shotts broth (Bullock, Hsu & Shotts 1986) was used to culture

F. columnare, *F. aquatile* and *F. johnsoniae*. The fatty acid methyl ester (FAME) profile was determined by the Microbial Identification System (version 4.5) of MIDI, Inc. (2002). Modified *Cytophaga* broth (Klesius, Lim & Shoemaker 1999) was used to culture *F. columnare*, *F. aquatile* and *F. johnsoniae*. The bacteria were grown at 28 °C for 24–48 h. Briefly, 100 ± 1 mg wet weight of cells were harvested and placed into 13 × 100 mm glass tubes. Sodium hydroxide and methanol solutions were added to the cells and the mixture was heated in a water bath for 30 min to saponify the cells. The fatty acids were methylated in hydrochloric acid and methanol reagents in a water bath at 80 °C for 10 min. The FAMES were extracted with hexane and methyl *tert*-butyl ether and washed with diluted sodium hydroxide. The FAMES were analysed on a Agilent 6850 gas chromatograph (25 m × 0.2 mm × 0.33 µm film thickness, Ultra 2 column) (Agilent, Palo Alto, CA, USA) using the CLIN40 method. The FAME results from the tilapia isolates, as well as six archived *F. columnare* strains from different origins (Table 1) were imported into the software program BioNumerics v. 3.0 (Applied Maths, Sint-Martens-Latem, Belgium) for analysis. A similarity matrix was created using Pearson product–moment correlation coefficient. Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA).

Molecular analysis

ISR analysis

Universal primers 16S-14F (5'-CTTGTACA CACCGCCCGTC-3', position 1389–1407 *E. coli* numbering) and 23S-1R (5'-GGGTTTCCCCA

Table 1 *Flavobacterium* spp. strains used in the study

Species	Strain	GenBank no.	Source	Geographic origin
<i>Flavobacterium columnare</i>	ATCC 23463 ^T	AY754372	Chinook salmon	Washington, USA
	MS-02-463	AY754384	Channel catfish	Mississippi, USA
	IR	AY754381	Common carp	Israel
	27	AY754360	Channel catfish	Alabama, USA
	BZ-1-02	AY753071	Nile tilapia	Brazil
	BZ-2-02	AY754375	Nile tilapia	Brazil
	BZ-4-02	AY754376	Nile tilapia	Brazil
	BZ-5-02	AY754377	Nile tilapia	Brazil
<i>Flavobacterium johnsoniae</i>	ATCC 17061 ^T	AY753067	Soil	England
<i>Flavobacterium aquatile</i>	ATCC 11947 ^T	AY753066	Deep well water	England

^T, Type strain.

TTCGGAAATC-3', position 124–110 *E. coli* numbering) against highly conserved regions were used to amplify the 16S-23S rDNA intergenic spacer region (ISR) (Zavaleta, Martinez-Murcia & Valera 1996). A single colony was resuspended in sterile water, boiled for 5 min, cooled on ice and briefly centrifuged. A quantity of 5 µL of supernatant were used as template DNA for polymerase chain reaction (PCR) amplification. PCR conditions were as described previously by Zavaleta *et al.* (1996). Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, Indianapolis, IN, USA) and sequenced by the Auburn University Genomics and Sequencing Laboratory. Calculation of similarity values and cluster analysis were carried out using the BioNumerics software taking into account the homologous nucleotide positions after discarding all unknown bases and gaps. A dendrogram was constructed with the same software package, using the UPGMA algorithm. Tree robustness was defined by a bootstrap analysis of 1000 replicates.

Experimental infection

In order to test the pathogenic potential of Brazilian isolates of *F. columnare* one strain was selected for experimental infection of channel catfish and Nile tilapia. Channel catfish fingerlings with a mean weight of 5.7 ± 1.35 g and mean length of 76.6 ± 5.41 mm were used. All assays were conducted in 57-L aquaria with flow through water at an exchange rate of 0.5 L h^{-1} at 28 °C. The channel catfish fingerlings were divided into three groups and challenged by the intramuscular (i.m.), intraperitoneal (i.p.) and bath immersion (20, 30 and 10 fingerlings, respectively) routes. Fingerlings from the i.m. group were challenged with 0.1 mL injection of 1×10^6 CFU mL⁻¹, from the i.p. group with 0.1 mL of a 1×10^8 CFU mL⁻¹ inoculum and the bath immersion group were exposed to a final concentration of 3×10^7 CFU mL⁻¹ in 3 L of water for 15 min. Control fish (the same number for each group) were injected with culture medium without bacteria (modified *Cytophaga* broth) for the i.m. and i.p. groups, or exposed to culture medium (10 mL) in a water bath for the same time. Clinical signs and mortality were recorded for 10 days post-challenge. For tilapia fingerlings only the i.m. route was evaluated and similar conditions were used, with some modifications, as follows: 20 fish with a

weight of 2.7 ± 0.84 g and mean length of 50.6 ± 6.04 mm, were injected with 0.1 mL of a 1×10^8 inoculum and the 20 control fingerlings were injected with the same volume of sterile culture medium. The groups were stocked in 36-L aquaria.

Results

Putative *F. columnare* strains were isolated from both fingerling and adult fish lesions. The colonies obtained were yellow, flat, with irregular (rhizoid) edges and strongly adhered to the agar surface. All isolates were Gram-negative long rods. From these, four strains, designated BZ-1-02, BZ-2-02, BZ-4-02 and BZ-5-02, three from adult fish and one from fingerlings, were phenotypically (biochemical tests, API ZYM and FAME analysis) and molecularly characterized (ISR sequencing). Tables 2 and 3 show the biochemical and enzymatic profiles of the selected isolates and reference strains used in this study, respectively. The phenotypic characteristics of Brazilian strains of *F. columnare* were identical to those reported by Bernardet, Segers, Vancanneyt, Berthe, Kersters & VanDamme (1996). All four identification methods used unequivocally ascribed the four tilapia isolates to the species *F. columnare*. All strains analysed presented the same biochemical and enzymatic properties similar to the reference strains of *F. columnare* ATCC 49512 and ATCC 23463^T. The isolates were not similar to *F. aquatile* (ATCC 11947^T) and *F. johnsoniae* (ATCC 17061^T) in terms of biochemical and enzyme profiles. FAME analysis showed a high similarity clustering of the tilapia isolates with other *F. columnare* isolates present in our database (Fig. 1). The major FAME components were 15:0 ISO (43%), 15:1 ISO G (14%), 17:0 ISO 3OH (10%), 15:0 ISO 3OH (7%), 17:1 ISO w9c (7%), 15:0 (1%), and 16:0 (1%). All *F. columnare* isolates clustered at 95% similarity. As the empirical cutoff point for this technique (90%) in our laboratory is below this level no intraspecific groups could be defined.

The PCR amplification of the ISR from Brazilian isolates yielded two fragments of approximately 400 and 600 bp. Both fragments were sequenced and showed homology with other *F. columnare* ISR sequences. This unique characteristic (other *F. columnare* isolates yield one ISR band of approximately 600 bp) might be explained by the presence of more than one operon type in the bacterial chromosome. However, as the 400 bp

Table 2 Biochemical profile of Brazilian strains of *Flavobacterium columnare*

Biochemical test	<i>F. columnare</i> Brazilian isolates ^a	<i>F. columnare</i> ATCC 23463	<i>F. aquatile</i> ATCC 11947	<i>F. johnsoniae</i> ATCC 17061
Flexirubin pigment	+	+	–	–
Congo red absorption	+	+	–	–
Cytochrome oxidase	+	+	–	+
Catalase	+	+	+	+
Nitrate reduction	–	–	–	–
Production of H ₂ S	+	+	+	+
Starch hydrolysis	+	+	+	+
Esculin hydrolysis	–	–	+	+
Gelatin hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Tyrosine hydrolysis	–	–	+	+
Arginine dihydrolase	–	–	–	–
Lysine decarboxylase	–	–	–	–
Ornithine decarboxylase	–	–	–	–
Tributyrin hydrolysis	+	+	+	+
Lecithin hydrolysis	+	+	–	+
Tween-20 hydrolysis	+	+	+	+
Chondroitin AC lyase	+	+	–	–
Acid from fructose	–	–	–	+
Acid from glucose	–	–	+	+
Acid from galactose	–	–	+	–
Acid from glycerol	–	–	–	+

^a Results from strains designated BZ-1-02, BZ2-02, BZ4-02 and BZ-5-02.**Table 3** Enzymatic profile (API ZYM test) of Brazilian strains of *Flavobacterium columnare*

Biochemical test	<i>F. columnare</i> Brazilian isolates ^a	<i>F. columnare</i> ATCC 23463	<i>F. aquatile</i> ATCC 11947	<i>F. johnsoniae</i> ATCC 17061
Alkaline phosphatase	+	+	+	+
Esterase (C4)	+	+	+	+
Esterase lipase (C8)	+	+	+	+
Lipase (C4)	–	–	–	+
Leucine arylamidase	+	+	+	+
Valine arylamidase	+	+	+	+
Cystine arylamidase	–	–	+	+
Trypsin	b	+	+	–
Chymotrypsin	–	–	–	–
Acid phosphatase	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+
α-Galactosidase	–	–	–	–
β-Galactosidase	–	–	+	+
β-Glucuronidase	–	–	–	–
α-Glucosidase	–	–	+	+
β-Glucosidase	–	–	+	+
N-acetyl-β-glucosaminidase	–	–	+	+
α-Mannosidase	–	–	+	–
α-Fucosidase	–	–	–	–

^a Results from strains designated BZ-1-02, BZ2-02, BZ4-02 and BZ-5-02.^b Strains BZ-1-02, BZ2-02 and BZ-5-02 were positive and strain BZ-4-02 was negative.

fragment showed a high sequence homology with the 600 bp, only the largest fragment was used for the analysis. Sequencing of the ISR confirmed *F. columnare* as the closest species to the tilapia isolates (Fig. 2). The four tilapia isolates presented two different types of ISR sequences but were more similar to each other than to other *F. columnare* isolates previously sequenced. The Brazilian isolates

clustered together at 77% similarity and shared only 60% sequence homology with other *F. columnare* isolates, including the type strain. Similarity levels with other *Flavobacterium* species were lower than 55%.

The strain selected for experimental infection (BZ-5-02) was pathogenic to both channel catfish and tilapia fingerlings. In channel catfish, there was

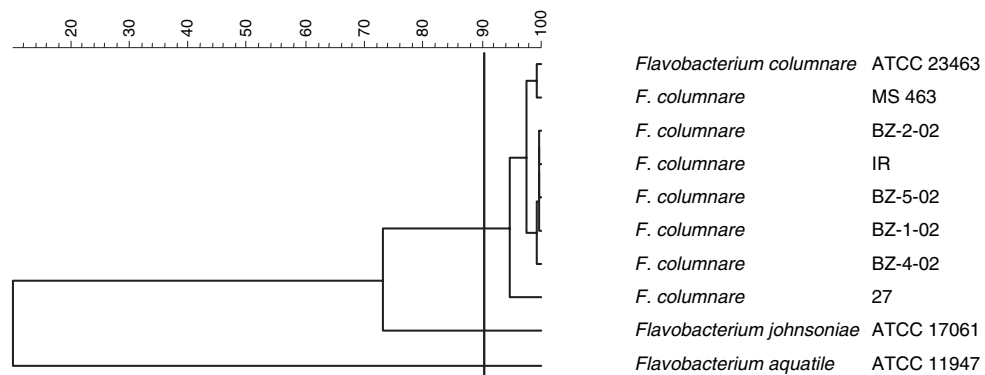


Figure 1 Dendrogram based on Pearson product-moment correlation analysis of fatty acid profiles of *Flavobacterium* spp. Vertical line indicates empirical cutoff point value.

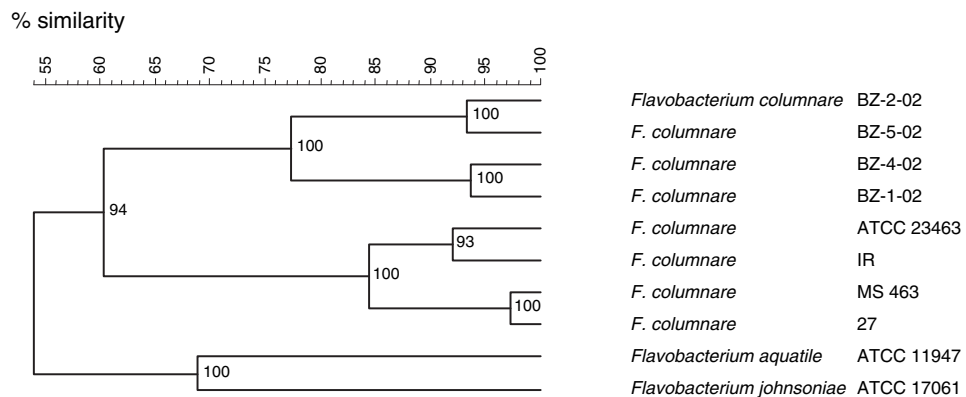


Figure 2 Phylogenetic tree based on ISR sequences of *Flavobacterium columnare* isolates analysed in the study. Numbers depict significant bootstrap values obtained for a bootstrap sampling of 1000.

a mortality rate of 80% by the i.m. route, 7% by the i.p. route and no mortality by bath immersion treatment. No unchallenged fingerlings developed clinical signs or died. In tilapia a mortality rate of 35% via the i.m. route was observed, with development of clinical signs of fin necrosis beginning at 2 days post-exposure. In unchallenged fingerlings a 5% mortality was observed, but without clinical signs of columnaris disease. *Flavobacterium columnare* was isolated from lesions from all morbid fish but was not isolated from all dead fish, presumably because of an overgrowth by environmental bacteria in culture medium.

Discussion

Fish production in Brazil has accelerated in recent years. The abundance of fresh water, and the tropical climate, have resulted in the increased cultivation of Nile tilapia. Despite these conditions,

the diagnosis of many important fish diseases, including columnaris disease, has not been completely achieved.

We isolated and characterized four strains of *F. columnare* from fingerling and adult Nile tilapia. To our knowledge this is the first report of isolation and characterization of *F. columnare* strains from Brazil. The results obtained from phenotypic and molecular analysis demonstrate the strong similarity within these strains. Furthermore, the FAME profiles were almost identical to those reported by Bernardet *et al.* (1996). However, the ISR sequence was not identical, indicating a certain degree of genetic diversity within these isolates. Based on the genetic data, a clonal linkage between outbreaks could not be inferred.

The results from experimental infections using channel catfish and Nile tilapia clearly demonstrate the pathogenic potential of this Brazilian strain of *F. columnare*. Channel catfish were susceptible by

the i.m. route and this may reflect the virulence of this pathogen for muscle tissue (Stringer-Roth *et al.* 2002). The lack of susceptibility of channel catfish to bath immersion may be associated with the absence of previous skin lesions in the fish, a factor known to stimulate the establishment of infection in experimental models with *F. columnare* (Bader, Nusbaum & Shoemaker 2003b). Although many reports of experimental infection with *F. columnare* exist (Amin, Abdallah, Faisal, Easa, Alaway & Alyan 1988; Soltani, Munday & Burke 1996; Decostere *et al.* 1998, 1999a; Decostere, Haesebrouck, Charlier & Ducatelle 1999b; Altinok & Grizzle 2001) using i.m. and bath immersion routes, there is no reliable protocol which permits a good reproducibility of results (Bader *et al.* 2003b) and this also causes difficulties in comparisons between experiments. Possible reasons for the significant lower susceptibility of Nile tilapia to i.m. infection compared with channel catfish may be because of differences in resistance between species, slight differences between experimental protocols and variations in the immune status of challenged fish.

References

- Altinok I. & Grizzle J.M. (2001) Effects of low salinities on *Flavobacterium columnare* infection of euryhaline and fresh-water stenohaline fish. *Journal of Fish Diseases* **24**, 361–367.
- Amin N.E., Abdallah I.S., Faisal M., Easa M.El-S., Alaway T. & Alyan S.A. (1988) Columnaris infection among cultured Nile tilapia *Oreochromis niloticus*. *Antonie van Leeuwenhoek* **54**, 509–520.
- Arias C.R., Welker T.L., Shoemaker C.A., Abernathy J.K. & Klesius P.H. (2004) Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish. *Journal of Applied Microbiology* **97**, 421–428.
- Bader J.A., Shoemaker C.A. & Klesius P.H. (2003a) Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*. *Journal of Microbiological Methods* **52**, 209–220.
- Bader J.A., Nusbaum K.E. & Shoemaker C.A. (2003b) Comparative challenge model of *Flavobacterium columnare* using abraded and unabraded channel catfish, *Ictalurus punctatus* (Rafinesque). *Journal of Fish Diseases* **26**, 461–467.
- Bernardet J.F., Segers P., Vancanneyt M., Berthe F., Kersters K. & VanDamme P. (1996) Cutting the Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family, Flavobacteriaceae, and proposal of *Flavobacterium hydatidis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *International Journal of Systematic Bacteriology* **46**, 128–148.
- Bullock G.L., Hsu T.C. & Shotts E.B. Jr (1986) Columnaris disease of fishes. *U.S. Fish and Wildlife Service Fish Disease Leaflet* **72**.
- Decostere A., Haesebrouck F. & Devriese L.A. (1998) Characterization of four *Flavobacterium columnare* (*Flexibacter columnaris*) strains isolated from tropical fish. *Veterinary Microbiology* **62**, 35–45.
- Decostere A., Haesebrouck F., Turnbull J.F. & Charlier G. (1999a) Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. *Journal of Fish Diseases* **22**, 1–11.
- Decostere A., Haesebrouck F., Charlier G. & Ducatelle R. (1999b) The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*). *Veterinary Microbiology* **67**, 287–298.
- Griffin B.R. (1992) A simple procedure for identification of *Cytophaga columnaris*. *Journal of Aquatic Animal Health* **4**, 63–66.
- Klesius P., Lim C. & Shoemaker C. (1999) Effect of feed deprivation on innate resistance and antibody response to *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus*. *Bulletin of the European Association of Fish Pathologists* **19**, 156–158.
- MIDI, Inc. (2002) *Sherlock® Microbial Identification System Operating Manual* (Version 4.5). MIDI, Inc., Newark, DE.
- Shamsudin M.N. & Plumb J.A. (1996) Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from four species of fish. *Journal of Aquatic Animal Health* **8**, 335–339.
- Shieh H.S. (1980) Studies on the nutrition of a fish pathogen *Flexibacter columnaris*. *Microbiology Letters* **13**, 129–133.
- Soltani M., Munday B.L. & Burke C.M. (1996) The relative susceptibility of fish to infections by *Flexibacter columnaris* and *Flexibacter maritimus*. *Aquaculture* **140**, 259–264.
- Stringer-Roth K.M., Yungchans W. & Caslake L.F. (2002) Differences in chondroitin AC lyase activity of *Flavobacterium columnare* isolates. *Journal of Fish Diseases* **25**, 687–691.
- Triyanto & Wakabayashi H. (1999) Genotypic diversity of strains of *Flavobacterium columnare* from diseased fish. *Fish Pathology* **34**, 65–71.
- Triyanto, Kumamaru A. & Wakabayashi H. (1999) The use of PCR targeted 16S rDNA for identification of genomovars of *Flavobacterium columnare*. *Fish Pathology* **34**, 217–218.
- Zavaleta A.I., Martinez-Murcia A.J. & Valera F.R. (1996) 16S-23S rDNA intergenic sequences indicate that *Leuconostoc oenos* is phylogenetically homogeneous. *Microbiology* **142**, 2105–2114.

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